The University of Chicago

I. The Preparation of Phenylethylbarbituric Acid

II. The Preparation of Para-Ureido-Phenylacetyl-urea, and Related Compounds

III. An Attempt to Filter the Enzymes of Milk

A DISSERTATION

SUBMITTED TO THE FACULTY OF THE OGDEN GRADUATE SCHOOL OF SCIENCE IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

(DEPARTMENT OF CHEMISTRY)

By Mary M. Rising

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I.

THE PREPARATION OF PHENYLETHYLBARBITURIC ACID.[1]

Phenylethylbarbituric acid is a member of the ureid group of hypnotic drugs, of which diethylbarbituric acid, introduced as "veronal" and now known as "barbital,"[2] is the best known and most important representative. Phenylethylbarbituric acid, "pheno-barbital,"[3] introduced as "luminal," is of specific value as a hypnotic and sedative in the treatment of epilepsy, and, therefore, is one of the group of essential synthetic remedies whose manufacture in this country has become a matter of importance in consequence of the war. In order to be in a position to advise and assist any would-be manufacturer with a minimum loss of time, the author of this paper (at the suggestion and under the direction of Dr. Stieglitz) studied the synthetic preparation of phenylethylbarbituric acid. Certain improvements in the method studied have suggested themselves and are reported for the benefit of any one preparing to supply this important synthetic.

The method of preparation of phenylethylbarbituric acid, which was investigated, is indicated in the following series of reactions:

(1) Ethyl oxalate is condensed with the ethyl ester of phenylacetic acid by means of sodium ethylate in order to form the sodium salt of the diethylester of oxalyl-phenylacetic acid.[4]

$$C_2H_5O_2C.CO_2C_2H_5 + H_2C(C_6H_5).CO_2C_2H_5 + NaOC_2H_5 \longrightarrow C_2H_5O_2C.C(ONa) : C(C_6H_5).CO_2C_2H_5 + C_2H_5OH. \quad (1)$$

W. Wislicenus,[4] who carried out this reaction, recommended as the condensing agent the use of pure sodium ethylate, which is prepared by the driving off of the excess of alcohol in a stream of inert gas (hydrogen) from a solution of sodium ethylate in ethyl alcohol.

In this stage of the synthesis two modifications have been found desirable. In the first place, one may do away with the laborious preparation of solid sodium ethylate and effect the combination in absolute ether solution by means of sodium wire and a few drops of absolute (ethyl or

[1] This paper was published in *J. Am. Chem. Soc.*, **40**, 725 (1918), by Mary Rising and J. Stieglitz.

[2] The official name of this drug, adopted by the U. S. Federal Trade Commission in the issuance of licenses for its manufacture, is "barbital." The reasons for the adoption of new official names for the licensed drugs and for the recommendation that these names be generally used by chemists and physicians in this country are presented in the *J. Am. Med. Assoc.*, **70**, 536 (1918).

[3] "Pheno-barbital" is the name for phenylethylbarbituric acid, which will be used in the issuance of licenses for its manufacture in this country.

[4] *Ber.*, **27**, 1092 (1894); *Ann.*, **246**, 315 (1888).

methyl) alcohol. A quantitative yield of the desired sodium salt is easily obtained. In the second place, the methyl esters may be substituted for the ethyl esters. Methyl oxalate is a solid and much more easily prepared than the ethyl ester, especially by the method finally adopted by us. The condensation of the methyl esters is, if anything, more smoothly effected than that of the ethyl esters.

(2) In the next stage of the synthesis, the dimethyl oxalylphenylacetic ester is converted by heat into the methyl ester of phenyl malonic acid by the loss of carbon monoxide, according to the method used by Wislicenus on the ethyl ester.

$$CH_3O_2C.CO.CH(C_6H_5)CO_2CH_3 \longrightarrow CO + C_6H_5CH(CO_2CH_3)_2. \quad (2)$$

Here too an advantage in the use of the methyl ester was observed in as much as methyl phenylmalonate is a solid which may be easily purified by recrystallization, whereas the ethyl ester is a liquid, which tends to decompose when distilled, even *in vacuo*.

(3) The ethylation of phenylmalonic methyl ester in alcoholic solution according to

$$C_6H_5CH(CO_2CH_3)_2 + NaOC_2H_5 + C_2H_5I \longrightarrow (C_6H_5)(C_2H_5)C(CO_2CH_3)_2 + NaI + HOC_2H_5 \quad (3)$$

leads to not altogether satisfactory yields as a result of saponification and decomposition of the ester in a parallel reaction, exactly as observed by Wislicenus for the ethyl ester.[1] But again the new methyl ester had the advantage of being a solid, which may be purified by recrystallization. The sodium salt of phenylmalonic methyl ester is easily prepared in ether suspension by treatment of the ester dissolved in absolute ether with sodium wire and a little alcohol. Ethylation of this salt in sealed tubes at 100° has been studied in the hope of improving the yield by avoiding the loss due to the saponifying action of sodium ethylate in alcoholic solution. The results thus far obtained have not been as satisfactory as expected but the effort will be continued, especially in the direction of experiments to be carried out at higher temperatures. The use of an autoclave would be easily compensated for by a greater yield of the ethylated product.

(4) The condensation of ethylphenylmalonic dimethylester with urea is the last stage in the synthesis of phenyl barbituric acid.

$$(C_6H_5)(C_2H_5)C(COOCH_3)_2 + CO(NH_2)_2 \longrightarrow (C_6H_5)(C_2H_5)\underline{C.CONHCONH.CO}. \quad (4)$$

According to the patent specifications[2] the condensation is effected by sodium ethylate in alcoholic solution by the heating of the mixture under

[1] *Ber.*, **28**, 815 (1895).

[2] U. S. Pat. 1,025,872 (1912).

a reflux condenser. This produced phenylethylbarbituric acid but only in exceedingly small quantity. A far better result was obtained by the method of condensation that Fischer[1] used in the preparation of diethylbarbituric acid, with the modification that sodium methylate in methyl alcohol was substituted for sodium ethylate in ethyl alcohol solution.

Further work on the improvement of the yields in the later stages of the synthesis of phenylethylbarbituric acid is in progress and other methods of accomplishing the synthesis especially with the aid of the Grignard reaction, are to be investigated.

Experimental Part.

1. Phenylacetic Methyl Ester, $C_6H_5CH_2CO_2CH_3$.—Phenylacetic methyl ester was prepared in general according to the directions of Wislicenus[2] for the preparation of the ethyl ester. To 200 g. of absolute methyl alcohol are added 100 g. of benzyl cyanide and 20 g. of water. The flask containing this mixture is placed under reflux, and dry hydrogen chloride gas passed into it at a rather rapid rate. The solution becomes hot, and after the gas is passed in for half an hour or so, a heavy white precipitate of ammonium chloride settles out with a rather violent reaction. The treatment with hydrogen chloride is continued to saturation, first without cooling, and then the mixture is cooled and saturated in the cold. The flask and contents are left standing for twenty-four hours, and are then heated for one hour on the water bath. The mixture is cooled, and 300 g. of ether and finally just sufficient water are added to it to dissolve the ammonium chloride when shaken in a separatory funnel. The ether solution is separated and washed with 10 cc. portions of sodium carbonate until the washings are alkaline. All the wash waters are combined, including that containing the ammonium chloride, and shaken with a little ether. This ether layer is separated, and all the ether solutions are combined, filtered, and dried over granulated calcium chloride. The oil remaining after the evaporation of the ether is fractionated. The yield of oil of boiling point[3] 212–216° (uncorr.) was 110 g., or 85.9% of the theoretical yield.

In this process, care was taken to wash the ether solution of the oil with only small amounts of sodium carbonate, in order to avoid hydrolysis of the ester by alkali. The alkali was then separated quickly and completely from the ether solution of the ester.

The course of the reaction may be summarized as follows:

Main reaction: $C_6H_5.CH_2.CN + CH_3OH + HCl \longrightarrow C_6H_5CH_2C(:NH_2Cl)OCH_3$ (5)

[1] *Ann.*, **335**, 338 (1904).

[2] *Ibid.*, **296**, 361 (1896).

[3] Radziozewski, *Ber.*, **2**, 208 (1869), gives the b. p. 220° for the ester prepared from phenylacetic acid in the usual way.

$C_6H_5.CH_2C(:NH_2Cl)OCH_3 + H_2O \longrightarrow NH_4Cl + C_6H_5.CH_2.CO_2CH_3$ (6)

By reactions: $C_6H_5.CH_2.CO_2CH_3 + HOH \longrightarrow$

$C_6H_5.CH_2CO_2H + CH_3OH$ (7)

and, to some extent: $C_6H_5CH_2C(:NH_2Cl)(OCH_3) \longrightarrow$

$C_6H_5CH_2CONH_2 + CH_3Cl$ (8)

2. Methyl Oxalate, $(COOCH_3)_2$.—Vanino's[1] method for the preparation of methyl oxalate was modified and the following far simpler method used with better results. In Vanino's method the sodium carbonate used rapidly saponifies the ester, lowering the yield.

To 378 cc. of absolute methyl alcohol are added 300 g. of dehydrated oxalic acid. The dehydration is accomplished sufficiently by the heating of the hydrated acid in shallow vessels in a steam oven overnight. The solution of oxalic acid in methyl alcohol is placed under reflux, and hydrogen chloride gas is passed into it in a strong stream. The mixture quickly becomes hot; it is then cooled in an ice bath to prevent decomposition of the ester. White crystals of methyl oxalate separate out slowly after hydrogen chloride has been passed in for some time and a solid mass of crystals eventually forms in the flask. The flask and contents are left standing for twenty-four hours, and the crystals are then brought on a funnel, and kept over suction for some hours to remove the hydrogen chloride. By drying the crystals further *in vacuo* for several days over sodium hydroxide or lime, the last traces of hydrogen chloride are removed. The melting point of the crystals was found to be 53.5°. The yield was 333 g., or 84% of the theoretical yield. This yield includes 28 g. recovered from the mother liquor when cooled to —15°.

Anschütz and Pictet's method,[2] used in the preparation of tartaric acid esters, and said to give quantitative yields for oxalic acid esters, was also tried. It gave very poor results and proved much more laborious than the above method.

3. Sodium Oxalylphenylacetic Methyl Ester, $CH_3O_2C.C(ONa):C(C_6H_5)CO_2CH_3$.—Wislicenus[3] obtained an 85% yield of the sodium salt of oxalylphenylacetic ethyl ester by the condensation of ethyl oxalate with phenylacetic ethyl ester, using alcohol-free sodium ethylate prepared in a stream of hydrogen as the condensing agent. We applied the Nef method of preparation of acetoacetic ester to this condensation, using sodium wire in an ether solution of the two esters, and adding a few drops of absolute methyl alcohol to start the reaction. Our yield ranged from 85 to 92% of the theoretical.

To 80 g. of sodium dried ether are added 4 g. of sodium wire and 20 g. of methyl oxalate. The oxalate dissolves only partially. There are then

[1] *Präparative Chemie*, **2**, 110 (1914).

[2] *Ber.*, **13**, 1178 (1880).

[3] *Ibid.*, **27**, 1092 (1894).

added 25 g. of phenylacetic methyl ester, and about ten drops of absolute methyl alcohol, and the mixture is placed at once under reflux. Soon after the alcohol is added, yellow crystals begin to form upon the sodium wire, and a flocculent precipitate appears in the ether solution. The sodium dissolves very slowly, with the evolution of hydrogen, and a mass of yellow precipitate gathers in the bottom of the flask, the liquid above it turning to a dark red. Two days are allowed for the completion of the reaction, and the mixture is then heated for one hour on the water bath. It sometimes happened that gentle heating on an electric oven was necessary to start the reaction, and then the precipitate came down rapidly. After the contents of the flask are cooled, the yellow precipitate of sodium oxalylphenylacetic methyl ester is brought upon a filter, washed with dry ether, and dried *in vacuo*. The yield varies from 37 to 40 g.

As the salt is very easily decomposed the product of the reaction must be kept dry. Our product was yellow, and perhaps not absolutely pure but attempts to purify it by solution of the salt in alcohol and precipitation with ether, as recommended by Wislicenus for the corresponding salt of the ethyl ester, failed, as the substance turned quickly to a soft, jelly-like mass.

A sodium determination showed that the salt is rather pure.

Subst. 0.7881; Na_2SO_4, 0.1919.
$C_{12}H_{11}O_5Na$. Calc. Na: 8.92%. Found: 8.77%.

The reaction is

$$(COOCH_3)_2 + C_6H_5CH_2CO_2CH_3 + NaOCH_3 \longrightarrow CH_3O_2C.C(ONa) : C(C_6H_5)CO_2CH_3 + 2CH_3OH. \quad (9)$$

4. Oxalylphenylacetic Methyl Ester, $CH_3O_2C.CO.CH(C_6H_5)CO_2CH_3$. —The sodium salt of oxalylphenylacetic methyl ester is treated with cold dilute sulfuric acid in excess, whereby an oil is formed. The oil is separated from the acid by extraction with ether, and the ether extract dried over calcium chloride.

After the evaporation of the ether, the ester is decomposed with the loss of carbon monoxide by being treated *in vacuo* in the manner described by Wislicenus[1] for the ethyl ester. The ester when warmed at 90° C. and 10 mm. shows evidence of decomposition, with evolution of a gas, and change of color of the oil from bright red to dark brown. The manometer shows a decided increase in pressure, and the bubbles of gas forming at the outlet of the pump may be ignited and burn with the blue flame characteristic of carbon monoxide. When the loss of carbon monoxide is complete, the pressure falls rapidly to the original 10 mm. The reaction is expressed in Equation 2 above.

5. Phenylmalonic Methyl Ester, $(CH_3OOC)_2CHC_6H_5$.—The distilla-

[1] *Ber.*, **27**, 1091 (1894).

tion *in vacuo* is continued as described in (4), until the thermometer rises to 120°. The small fraction of impure ester passing over below this point is collected separately. At 120° crystals of phenylmalonic methyl ester form at the tip of the water condenser and in the receiving vessel, and at this time the distillation is interrupted, and the liquid in the flask transferred to an Anschütz distilling flask. The distillation is then resumed, after the pressure is again brought to 10 mm., and the main fraction of the oil distilling between 147° and 159° is collected. This entire distillate crystallizes readily.

The purification of the ester is accomplished by recrystallization from ligroin of low boiling point (40–50°) from which rosets of white crystals separate, when the solvent is allowed to evaporate spontaneously. The melting point of pure crystals was found to be 50°. The ester was identified by analysis.[1]

Subs. 0.1555; CO_2, 0.3628; H_2O, 0.0813.
Calc. for $C_{11}H_{12}O_4$: C, 63.46%; H, 5.82%. Found: C, 63.64%; H, 5.85%.

The crude yield from 78 g. of the sodium salt of oxalylphenylacetic ester was 42.2 g., or 67% of the theoretical yield.

6. Phenylethylmalonic Methyl Ester, $(CH_3OOC)_2C(C_2H_5)(C_6H_5)$.—Wislicenus[2] describes the alkylation of phenylmalonic ethyl ester with methyl iodide in the presence of sodium methylate. He reports a yield of the pure ester, an oil, which was 60% of the theoretical. Our experience with the ethylation of the methyl ester by the method of Wislicenus (described in detail for malonic esters by Conrad and Limpach),[3] was discouraging with regard to yields, which were very low.

Sodium methylate is prepared from 30 g. of absolute methyl alcohol and 3 g. of sodium. To this solution, 30 g. of phenylmalonic methyl ester are added, and the flask containing the mixture is placed under reflux. After solution of the ester, which occurs readily, 30 g. of ethyl iodide are added in small portions through the condenser tube. There is no apparent reaction, and it is necessary to heat the mixture for three or four days over a water bath in order to bring about the ethylation. That the reaction does not go to completion is shown by the fact that the mixture in the flask remains alkaline. It was, therefore, necessary to neutralize it before proceeding further with the preparation of the ester.

At this point the directions of Conrad and Limpach were modified, with good results. The residue left in the flask after being heated for several days is made neutral, or slightly acid, with dilute sulfuric acid, and enough water is added to dissolve the sodium iodide formed in the re-

[1] I am indebted to Mr. R. Q. Brewster for this combustion and for the others reported on in this paper.

[2] *Ber.*, **28**, 815 (1895).

[3] *Ann.*, **204**, 129 (1880).

action, and to cause the ethylated ester to separate in an oily layer at the bottom of the flask. This oil is separated from the water above it, and dried over calcium chloride. The aqueous layer contains a small amount of the oil, and is extracted twice with ether, and the ether solution is dried over calcium chloride. After the evaporation of the ether, the remaining oil, which is dark red in color, is added to the main portion, and distilled *in vacuo*.

From 80 to 90°, at 10 mm., there is obtained a dark red oil and some purple iodine fumes are formed. This change is complete below 100° and a pale yellow liquid remains in the distilling flask. From 100 to 135°, a fraction of impure ethylated ester is obtained, and from 135 to 155°, the main fraction of the oil distils.

The fraction of the higher boiling point crystallizes when cooled, and remains solid at room temperature. The fraction of the lower boiling point crystallizes slowly on cooling, but softens somewhat at room temperature. The yield of the two fractions before purification was 26 g. or 76% of the theoretical, and the crystals showed a melting point of 33°. By recrystallization from ligroin of low boiling point, crystals were obtained with a melting point of 37°. The crystals obtained by spontaneous evaporation of the solvent were large, colorless, and transparent.

The ethylated ester was identified by analysis:

Subs. 0.1544; CO_2, 0.3750; H_2O, 0.0954.
Calc. for $C_{13}H_{16}O_4$: C, 66.17; H, 6.83. Found: C, 66.24; H, 6.91.

The reaction is expressed in Equation 3 above.

7. Phenylethylbarbituric Acid, $(C_6H_5)(C_2H_5)C.CONHCONHCO$.—The condensation of the phenylethylmalonic methyl ester with urea to form phenylethylbarbituric acid was first attempted by the method described in the patents. The ester and urea are heated under reflux for six hours, with sodium alcoholate as the condensing agent. By the use of this method, we obtained only a very small amount of phenylethylbarbituric acid, and the yield was too low to warrant the use of the method for practical purposes.

The method of Fischer[1] for the preparation of diethylbarbituric acid, was followed next, with much better success and when carried out on a large scale, this method would undoubtedly give a higher yield than was obtained by us in working with small amounts of material.

Sodium methylate is prepared from 14.2 g. of absolute methyl alcohol and 0.9 g. of sodium. To it are added 2.5 g. of phenylethylmalonic methyl ester and 1.1 g. of urea. This mixture is heated in a sealed tube at 105–108°, for six hours. When the tube has cooled, the white precipitate

[1] *Ann.*, **335**, 338 (1904).

(chiefly of sodium carbonate) is removed and the filtrate is again heated in a sealed tube for six hours at 105–108°. Any further precipitate of sodium carbonate which has formed during the second heating is removed as before. The filtrate, which is alkaline, is neutralized with acetic acid, and the alcohol evaporated *in vacuo*. When the last of the alcohol is gone, the residue in the distilling flask has an oily appearance, and when a few cubic centimeters of water are added, a cloudy emulsion of the oil and water forms. This emulsion is now extracted with ether, and the ether allowed to evaporate spontaneously. The residue is a mass of crystals in a thick oil, and is a mixture of phenylethylbarbituric acid and unchanged ester. It is extracted with ligroin of low boiling point, which dissolves the ester. The remaining white mass is purified by recrystallization from boiling water, and after two recrystallizations, the product shows a melting point of 174°. The crystals are pure white, and lustrous. The yield was close to a gram or about 40% of the theoretical. The reaction is given in Equation 4 above.

II.

THE PREPARATION OF PARA-UREIDO-PHENYLACETYLUREA AND RELATED COMPOUNDS.[1]

A study of the preparation of ureido-phenylacetylurea was begun by the author, with the aid and advice of Professor Stieglitz, following the publication some time ago of the work upon the synthesis of phenylethyl-barbituric acid.[2] Phenylethyl-barbituric acid (luminal), widely used in the treatment of epilepsy, has hypnotic and sedative properties. Under certain conditions, the drug is known to have a toxic effect, and for this reason its use cannot be prescribed in all cases. With time of peace at hand, when there is leisure to work along lines of theory, rather than along those laid down by the imperative demands of war needs, the building up of a new series of compounds, related chemically to phenylethyl-barbituric acid, was undertaken. The effort has been made to retain in these compounds the excellent hypnotic properties of phenylethyl-barbituric acid, avoiding meanwhile those properties which are harmful. The compounds prepared in this way are to be thoroughly tested physiologically, in the hope of discovering a hypnotic of even greater value than is phenylethyl-barbituric acid.

The fact that phenylethyl-barbituric acid possesses toxic properties which are not possessed by the closely related compound, diethyl-barbituric acid, suggested the toxicity of the phenyl group. For the modification of the phenyl group, we have first tried the introduction in the phenyl radical of a urea group, which might at the same time enhance the hypnotic properties. Further, in place of the use of a dicarboxylic acid radical as in diethyl-barbituric acid (veronal), and in phenylethyl-barbituric acid, a monocarboxylic acid, phenylacetic acid, was first used, as in the case of the hypnotic diethylbromo-acetylurea (adalin), as giving products easier of approach. Finally, we are introducing into our compounds ethyl groups, and bromine, which have, as is well known, hypnotic properties. These considerations have led us to undertake the preparation of the following group of compounds: (1) *p*-Ureido-phenyl-acetylurea. (2) *p*-Ureido-phenylethyl-acetylurea. (3) *p*-Ureido-phenyldiethyl-acetylurea. (4) *p*-Ureido-phenylethyl-bromoacetylurea. The preparation of the first of these compounds is described in this paper.

p-Ureido-phenyl-acetylurea was made on the basis of the following series of reactions:

(1) *p*-Nitrophenyl-acetyl chloride is condensed with urea in boiling benzene, and *p*-nitrophenyl-acetylurea is formed.[3]

[1] This paper was published in *J. Am. Chem. Soc.*, **42**, 128 (1920).

[2] Rising and Stieglitz, *Ibid.*, **40**, 4, 725 (1918).

[3] Jacobs and Heidelberger, *Ibid.*, **39**, 2418 (1917).

$NO_2.C_6H_4.CH_2.COCl + NH_2.CO.NH_2 \longrightarrow$
$NO_2.C_6H_4.CH_2.CO.NH.CO.NH_2 + HCl.$

(2) *p*-Nitrophenyl-acetylurea is reduced quantitatively to *p*-aminophenyl-acetylurea by means of stannous chloride in glacial acetic acid in the cold.[1]

$2NO_2.C_6H_4.CH_2.CO.NH.CO.NH_2 + 6SnCl_2 + 14HCl \longrightarrow$
$(H_2N.CO.NH.CO.CH_2.C_6H_4:NH_3)_2SnCl_6 + 5SnCl_4 + 4H_2O.$

(3) The hydrochloride of *p*-aminophenyl-acetylurea is treated with potassium cyanate, whereupon rearrangement takes place, and *p*-ureidophenyl-acetylurea is formed.

$ClH_3N.C_6H_4.CH_2.CO.NH.CO.NH_2 + KCNO \longrightarrow$
$NH_2.CO.NH.C_6H_4CH_2.CO.NH.CO.NH_2 + KCl.$

Some preliminary preparations of related urea derivatives are first to be described:

(1) The hydrochloride of *p*-aminobenzyl cyanide was treated with potassium cyanate, and *p*-ureidobenzyl cyanide was formed.

$ClH_3N.C_6H_4.CH_2.CN + KCNO \longrightarrow NH_2.CO.NH.C_6H_4.CH_2.CN + KCl.$

(2) The methyl ester of *p*-ureido-phenylacetic acid is prepared: (*a*) by the esterification of *p*-ureidobenzyl cyanide,

$NH_2.CO.NH.C_6H_4.CH_2.CN + HOCH_3 + H_2O + HCl \longrightarrow$
$NH_2.CO.NH.C_6H_4.CH_2.COOCH_3 + NH_4Cl,$

and, (*b*) by the action of the hydrochloride of *p*-amino-phenylacetic methyl ester with potassium cyanate.

$ClH_3N.C_6H_4.CH_2.CO.OCH_3 + KCNO \longrightarrow$
$NH_2.CO.NH.C_6H_4.CH_2.CO.OCH_3 + KCl.$

(3) By the hydrolysis of *p*-ureido-phenylacetic methyl ester, *p*-ureido-phenylacetic acid is obtained.

The ethylation of benzyl cyanide, preliminary to the preparation of *p*-ureido-phenylethyl-acetylurea and the other compounds listed above, will be discussed below in connection with the preparative details.

Experimental Part.

Benzyl cyanide was converted into *p*-nitrobenzyl cyanide according to the method of Pschorr, Wolfes, and Buckow.[2] The method of these authors, together with certain modifications suggested by Hanke,[3] was

[1] Weyl, *Die Methoden der organischen Chemie*, **2**, 211 (1909); cf. Thiele and Dimroth, *Ann.*, **305**, 114 (1899). The first product isolated (see below), is the chlorostannate. Jacobs and Heidelberger (*Loc cit.*), effected this reduction with ferrous sulfate and ammonia.

[2] *Ber.*, **33**, 17 (1900).

[3] Dr. Milton Hanke's paper including this preparation is shortly to appear in *J. Am. Chem. Soc.*

used for the reduction of nitrobenzyl cyanide. *p*-Aminobenzyl cyanide, prepared in this way, was used in the synthesis of the first of our ureids.

1. ***p*-Amino-phenylacetic Methyl Ester,** $p\text{-}NH_2.C_6H_4.CH_2.CO.OCH_3$.— The hydrochloride of the ester is prepared according to the method used by us for the preparation of phenylacetic methyl ester in the synthesis of phenylethyl-barbituric acid.[1] To a mixture of absolute methyl alcohol (112 g.), and water (6 g.) is added *p*-aminobenzyl cyanide (28 g.). When the saturation with hydrogen chloride is begun, the hydrochloride of *p*-aminobenzyl cyanide is precipitated. As the reaction progresses, the hydrochloride goes into solution gradually, and after some time a precipitate of ammonium chloride appears. When the process of esterification is complete, the alcoholic solution is cooled, decanted from the ammonium chloride, and almost all the alcohol evaporated *in vacuo*. On cooling, a heavy mass of the hydrochloride of *p*-amino-phenylacetic methyl ester settles out and is collected on a filter. The crude yield from 28 g. of cyanide was 32 g., containing some ammonium chloride. It was purified in the following way: A mixture of one part of absolute alcohol and 4 parts of dry ether was prepared, and 60 cc. of this solution shaken with 3 g. of the impure hydrochloride in a separatory funnel. All but a small sediment of ammonium chloride dissolved, and the supernatant solution of the hydrochloride was siphoned off. This solution was evaporated to $^1/_2$ its volume *in vacuo*, the residue cooled, and a heavy mass of nearly white, silvery leaves obtained, which is the hydrochloride of *p*-amino-phenylacetic methyl ester. An analysis for chlorine showed the product to be pure. The analysis of the hydrochloride of this same ester as prepared by Salkowski[2] showed a very high chlorine content.

Subs., 0.1095: 0.1*N* $AgNO_3$, factor 0.967, 5.59 cc.
Calc. for $C_9H_{12}O_2NCl$: Cl, 17.60. Found: 17.50.

The hydrochloride was dissolved in 25 cc. of water and the solution made just alkaline with sodium hydrogen carbonate, methyl orange being used as indicator. The solution was then titrated with silver nitrate in the usual way, potassium chromate being used as indicator.

The free base was prepared from the hydrochloride as follows: The hydrochloride (4 g.) was dissolved in 20 cc. of water and 50 cc. of ether was added. The calculated amount of sodium hydrogen carbonate (2.1 g., dissolved in 30 cc. of water) was added in small portions, and the whole shaken in a separatory funnel. The ether layer was separated, and the aqueous residue extracted twice with ether. The ether extractions were combined and dried over sodium sulfate, the ether evaporated, and the remaining oil fractionated. At 75°, and 20 mm. pressure, the oil commenced to boil. At 140–150° and 17 mm. pressure the main fraction,

[1] *Loc. cit.*
[2] *Ber.*, **28**, 1919 (1895).

the ester itself, distilled. At 155° and 17 mm. pressure a last fraction was obtained. Salkowski gives no boiling point in his description of the amino ester.

2. ***p*-Ureidobenzyl Cyanide,** p-$NH_2.CO.NH.C_6H_4.CH_2CN$.—The hydrochloride of *p*-aminobenzyl cyanide is prepared by precipitation with dry hydrogen chloride from the solution of the free base in dry ether. The hydrochloride (13 g.), obtained in this way, is dissolved in 75 cc. of water, and to this solution is added potassium cyanate (7 g., the calculated amount and $^1/_3$ excess, dissolved in 24 cc. of water). There is slight effervescence, due to the formation of cyanic acid, and the solution thickens to a pasty mass as the ureido compound forms. This mixture is evaporated to dryness on the water bath, extracted with water to remove potassium chloride formed in the reaction, and any unchanged potassium cyanate, and the undissolved residue is collected on a filter. Recrystallization of this residue from 95% alcohol yielded a product melting sharply at 170.5°.

Subs., 0.0514: 10.92 cc. N (over 50% KOH) at 22° and 752.5 mm. (24°).
Calc. for $C_9H_9N_3O$: N, 24.00. Found: 23.91.

3. ***p*-Ureido-phenylacetic Methyl Ester,** p-$NH_2.CO.NH.C_6H_4.CH_2.CO.OCH_3$.—This compound was prepared in two ways: (*a*) by the esterification of *p*-ureidobenzyl cyanide, and (*b*) from *p*-amino-phenylacetic methyl ester hydrochloride by treatment with potassium cyanate.

(*a*) *Esterification of p-Ureidobenzyl Cyanide.*—The ureidobenzyl cyanide (7 g.) is dissolved in a mixture of absolute methyl alcohol (42 g.) and water (2 g.). The process of esterification used in the preparation of *p*-amino-phenylacetic methyl ester is carried out. When the alcoholic solution is decanted from the ammonium chloride formed in the reaction, it is set aside for a few hours, and presently a precipitate of white crystals of the ureido ester separates. The crystals are collected on a filter and are found to melt at 110°. The yield of crude material was 4.5 g. Recrystallization from 95% alcohol gave a product melting at 132°.

(*b*) *Action of Potassium Cyanate on p-Amino-phenylacetic Methyl Ester Hydrochloride.*—The hydrochloride (6 g.) is dissolved in water (48 cc.) and potassium cyanate (4.2 g., the calculated amount and $^1/_3$ excess, dissolved in 10 cc. of water) is added. The reaction seemed to go to completion at once, and the pasty product of the reaction was not evaporated to dryness as in other similar preparations. When evaporation was carried out over the water bath, ammonia fumes were given off, showing decomposition. The mass was allowed to stand in the cold for about an hour, and was then collected on a filter and washed thoroughly with water. A yield of 5.6 g. of crude ester was obtained. It was recrystallized by solution in the smallest possible amount of warm 95% alcohol,

and precipitation from this solution with a large volume of ether. White, shining needles were obtained in this way, of melting point 131–132°.

Subs., 0.1259: 18.22 cc. N (over 50% KOH), at 19° and 749.2 mm.
Calc. for $C_{10}H_{12}N_2O_3$: N, 13.46. Found: 13.46.

4. ***p*-Ureido-phenylacetic Acid,** *p*-$NH_2.CO.NH.C_6H_4.CH_2.COOH$.—This acid was first prepared by Traube.[1] Our preparation by the hydrolysis of *p*-ureido-phenylacetic methyl ester and recrystallization from alcohol gave a purer product. It melted with decomposition at 184–185°. (Traube, m. p. 174°.)

Subs., 0.1285: 17.00 cc. N (over 50% KOH) at 28° and 747.3 mm. (28°).
Calc. for $C_9H_{10}N_2O_3$: N, 14.43. Found: 14.32.[2]

The Preparation of *p*-Ureido-phenyl-acetylurea.

1. ***p*-Nitro-phenylacetic Acid,** *p*-$NO_2.C_6H_4.CH_2.COOH$.—The acid was prepared according to the method of Gabriel.[3] A quantitative yield was obtained, m. p. 151°. (Gabriel, m. p. 151.5–152°.)

2. ***p*-Nitro-phenylacetyl Chloride,** *p*-$NO_2.C.H_4.CH_2COCl$.—*p*-Nitro-phenylacetic acid, prepared as indicated above, was converted into its chloride by the method described by Wedekind.[4] A quantitative yield of *p*-nitro-phenylacetyl chloride was obtained, m. p. 42–45°, which was considered sufficiently pure for use. (Wedekind, m. p. 47°.)

3. ***p*-Nitro-phenyl-acetylurea,** *p*-$NO_2.C_6H_4.CH_2.CO.NH.CO.NH_2$.—*p*-Nitro-phenylacetyl chloride was converted into *p*-nitro-phenyl-acetyl-urea by treatment with urea in benzene solution.[5] When the reaction was complete the *p*-nitro-phenyl-acetylurea was collected on a filter. The crude material turned brown at 220° and melted with decomposition at 230°. Its purification was accomplished by recrystallization from warm, glacial acetic acid, or from amyl alcohol. The purified product turned slightly yellow at 230°, softened somewhat at 233°, and melted to a reddish brown liquid at 237–239°.[6] This liquid solidified on cooling, and when put in the bath again at 225°, melted.

Subs., 0.1027: 17.73 cc. N (over 50% KOH) at 26° and 746 mm. (23°).
Calc. for $C_9H_9O_4N_3$: N, 18.83. Found: 18.84.

4. ***p*-Amino-phenyl-acetylurea,** *p*-$NH_2.C_6H_4.CH_2.CO.NH.CO.NH_2$.—The reduction of *p*-nitro-phenyl-acetylurea was accomplished with stannous chloride in glacial acetic acid which was saturated with hydrogen

[1] *Ber.*, **15**, 2121 (1882).
[2] Traube found 14.04% *N*.
[3] *Ber.*, **15**, 834 (1882).
[4] *Ann.*, **378**, 289 (1911).
[5] Later we found that Jacobs and Heidelberger (*Loc. cit.*) had prepared this compound by practically the same method.
[6] As decomposition occurs at this temperature, the melting point depends largely on the speed with which the bath is heated. Jacobs and Heidelberger give the melting point as 250–2°, with complete decomposition.

chloride. The following method was found to be very successful, with each step in the process well defined: Its main advantage lies in the fact that the reduction takes place in the cold. The urea derivative had shown itself to be rather unstable when kept at a high temperature for any length of time. Dry hydrogen chloride was passed into 60 cc. of glacial acetic acid to saturation. To this acid was added 3 mols. of crystallized stannous chloride and 50% excess, in all, 11.6 g. of 85% stannous chloride being used. The acid was heated slightly to dissolve the stannous chloride. The mixture was then cooled and *p*-nitro-phenyl-acetylurea (2 g.) added, and a stream of dry hydrogen chloride led into the flask, while the contents of the flask were stirred gently with the aid of a motor. After about 10 minutes, the urea was dissolved completely. Within 15 minutes more a heavy, white precipitate settled out of the solution, which proved to be the complex tin salt of the amine. The flask meanwhile became somewhat warm from the heat of reaction. The stirring was continued till the mixture was quite cool (for about one hour). At the end of that time the tin salt was collected. When dry, the yield of this salt was 4.1 g., the mass probably containing some stannous chloride, and the crystals being somewhat solvated.

The free base was obtained as follows: The tin salt was dissolved in water and the solution made sufficiently acid with hydrochloric acid to give a blue-green tint with methyl violet.[1] Tin sulfide was then precipitated with hydrogen sulfide, about an hour and a half being used for a complete precipitation. The stannic sulfide was removed by filtration and the filtrate concentrated *in vacuo* at 45° to a small volume. It was then made slightly basic with ammonia when a heavy precipitate of slender, white needles settled out. The base thus formed may be recrystallized by solution in acid and reprecipitation with ammonia, or it may be recrystallized from boiling water, in which it is fairly soluble. It does not melt, but decomposes at 192–3°. The yield of the pure compound was 1.2 g., or 70% of the theoretical amount.[2]

Subs., 0.0551: 10.75 cc. N (over 50% KOH) at 21.5° and 748.5 mm. (24°).
Calc. for $C_9H_{11}O_2N_3$: N, 21.76. Found: 21.83.

5. ***p*-Ureido-phenyl-acetylurea,** p-$NH_2.CO.NH.C_6H_4.CH_2.CO.NH.CO.NH_2$.—The hydrochloride of *p*-amino-phenyl-acetylurea was prepared by solution of the base (0.5 g.) in *N* hydrochloric acid (2.57 cc.). To this solution of the hydrochloride was added a solution of 0.28 g. of potassium cyanate (1 mol. and $^1/_3$ excess) in 10 cc. of water. After the addition of the cyanate, the mixture became at once thick and pasty, and was evaporated to dryness on the water bath. The residue was then taken up in a little water to remove the potassium chloride formed in the

[1] Stieglitz, *Qualitative Analysis*, 1, 214 (1913).

[2] Jacobs and Heidelberger effected the reduction with ferrous sulfate. Their crude yield was 73% of the theoretical amount.

reaction and the solid collected on a filter. The ureido-ureid was found to be quite soluble in hot water and is recrystallized from it. The yield of purified product was 0.47 g., or 77.5% of the theoretical amount.

Subs., 0.0816: 17.40 cc. N (over 50% KOH) at 21° and 745 mm. (22°).
Calc. for $C_{10}H_{12}O_3N_4$: N, 23.72. Found: 23.79.

The Ethylation of Benzyl Cyanide.

α-Phenylbutyronitril, $CH_3.CH_2.CH(C_6H_5).CN$.—The substitution of an alkyl group for one of the aliphatic hydrogens in the benzyl cyanide molecule takes place in two stages, the formation of a sodium salt and the replacement of sodium by the ethyl group.[1]

The completeness of the reaction in which α-phenylbutyronitril is formed depends, first of all, clearly upon the formation of a maximum amount of the sodium salt of benzyl cyanide. In the first attempt to prepare this sodium salt, metallic sodium was used, but the method of Bodroux[2] for the preparation of α-phenylbutyronitril was otherwise followed. Bodroux used sodium amide in place of metallic sodium.

Fine sodium wire (2.4 g.) is placed in 40 cc. of sodium-dried ether, under a reflux condenser. Benzyl cyanide (12 g.) is then dropped slowly into the flask containing the sodium and ether, two or three drops of absolute alcohol are added, and the reaction begins promptly. The ether boils, and most but not all of the sodium dissolves in the course of an hour. At the end of that time the ether solution has turned red, and a mass of the sodium salt, mixed with small particles of sodium, is deposited on the bottom of the flask. The mixture is heated for half an hour over the water bath, and then cooled. Ethyl iodide (16 g.) is added slowly to the suspension of the sodium salt, whereupon the mixture becomes warm and boils, and a light colored precipitate (NaI) settles out. When the boiling has ceased, the contents of the flask are heated for half an hour over the water bath and are then treated with a little water to dissolve particles of sodium and the sodium iodide. The ether layer is separated and combined with ether extracts of the aqueous solution. The whole ether extract is dried with calcium chloride and the ether removed by evaporation. The residue from the ether solution is a dark red oil, which, when subjected to vacuum distillation, boils from 70–105°, at 10 mm. pressure, all but a few drops of the distillate passing over above 100°. The yield of distillate was 6.4 g.

When sodium amide[2] was used in the preparation of the sodium salt of benzyl cyanide, a better yield of the ethylated product was obtained. From 12 g. of benzyl cyanide, a yield of 10 g. of distillate boiling at 108–118°, at 12 mm., was obtained. This oil contains, as stated by Bodroux,

[1] *Bull. soc. chim.*, **4**, Ser. 7, 666–667 (1910).
[2] Bodroux, *Loc. cit.*

α-phenylbutyronitril, and any unchanged benzyl cyanide Since the boiling points of the two nitrils lie too close together to make a separation by fractionation possible, it was found necessary by us to saponify the nitrils, in order to effect a separation. Phenylacetic acid melts at 76°, and α-phenylbutyric acid at 42°. In order to determine the degree of completeness of the reaction just described, hydrolysis of the distillate of boiling point 108–118°, at 12 mm., was undertaken.

Our method of saponification was the following: The 10 g. of distillate was boiled for 8 hours with 15 g. of potassium hydroxide in 60 g. of 95% alcohol. At the end of this time the alcohol was distilled off *in vacuo* and the residue was extracted with ether to remove any unchanged nitrils. The alkaline solution was then made slightly acid with dil. hydrochloric acid and extracted with ether several times. The ether extracts were combined, dried over calcium chloride, and the ether evaporated. The residue, a thick oil, solidified slowly on standing *in vacuo*, and 10.3 g. of crystals were obtained melting at 37–40°. The crystals were mixed with some oily material, which was removed by purification. To this end, the oily crystals were dissolved in concentrated ammonia and the solution was cooled in a freezing mixture. To it was added slowly cold conc. hydrochloric acid and enough water to dissolve the ammonium chloride which formed. When an excess of acid had been added, the solution became turbid, and if it had been kept at a low temperature during the addition of the acid, the α-phenylbutyric acid precipitated out in fairly pure form. Crystals obtained in this way were slightly yellow and melted at 40–42°. From 12 g. of benzyl cyanide, a yield of 8.1 g. was obtained, or 48% of the theoretical amount.

From a second preparation of α-phenylbutyronitril with sodium amide, in which 48 g. of benzyl cyanide was used, a yield of 49 g. of distillate boiling at 70–110°, at 10 mm., was obtained, all but a few drops of which distilled above 100°.

The distillate (6.4 g.) from the ethylation of 12 g. of benzyl cyanide with metallic sodium was hydrolyzed and a yield of about 4 g. of α-phenylbutyric acid obtained, of melting point 41–42°.

An attempt to substitute calcium carbide for sodium amide and sodium in the action with benzyl cyanide was made, but proved unsuccessful, because the carbide was not attacked appreciably.

Summary.

The successful preparation of *p*-ureido-phenyl-acetylurea is described as the first of a series of ureidophenyl acyl derivatives of urea which are intended to form the basis for a study of their physiological activity, par-

ticularly as hypnotics. Intermediate products and their preparation are described. The preparation of other derivatives of the series, such as ureido-phenylethyl-acetylurea, and ureido-phenylethyl-barbituric acid, is well under way in this laboratory and will be reported on at a later date.

The writer wishes to express her appreciation of the generous assistance and helpful suggestions given by Professor Stieglitz in connection with this work.

III.

AN ATTEMPT TO FILTER THE ENZYMES OF MILK.[1]

Milk, cow's as well as human, contains certain enzymes of unknown origin and chemical constitution.[2] Since nature provides these compounds in natural foods for the young it is safe to assume that they have their important uses, although unknown to us.

If this conclusion is warranted, the value of a sterilized milk with active enzymes is apparent. For artificial feeding of infants boiled, "pasteurized" or "certified" milk is used. Boiled milk contains no enzymes, these being destroyed at a temperature of 72–80°. Milk, pasteurized for 30 minutes at 60°, gives a positive test for enzymes; but the milk is not filtered and suspended dirt remains in it, and there is no certainty that all the dangerous germs and their spores are killed at the relatively low temperature. Certified milk is obtained by methods in which special precautions are observed, but such milk contains, nevertheless, bacteria which multiply rapidly. Milk sterilized by filtration would be free from germs and suspended impurities and any change of the enzyme would be avoided.

With this ultimate aim the experiments described in this paper were undertaken for the purpose of discovering a method of filtering milk enzymes. Repeated attempts by us to accomplish this have met with failure, and no filter has been found with pores large enough to allow the passage through them of the colloidal enzyme body, without at the same time allowing the passage of bacteria. The results obtained and observations made should, however, prove of value to these interested in this problem.

Certified milk about 12 hours old was used in our experiments. We tested only for the Shardinger enzyme.[3] The reaction for this in milk is discussed by Wieland[4] in his paper on catalyzing agents. This reaction, upon which all of our experiments and conclusions depend, is as follows: If to milk methylene blue and salicylic aldehyde are added then in presence of the Schardinger enzyme, which is a catalytic agent, the salicylic aldehyde will reduce and decolorize the methylene blue and be itself oxidized to salicylic acid. When the enzyme is destroyed by boiling or other means, the oxidation reduction reaction takes place so slowly, that it is not perceptible at all.

[1] This paper was published by J. F. Piccard and Mary Rising in the *J. Am. Chem. Soc.*, **40**, 1275 (1918).

[2] J. König, "Chemie der menschlichen Nahrungs und Genussmittel.," **3**, pp. 238–9.

[3] F. Schardinger, *Z. Unters, Nahr. Genussm.*, **5**, 1113 (1902); *Chem. Zentr.*, **1**, 98 (1903); *Chem. Ztg.*, **28**, 704 (1904); *Chim.*, **2**, 737 (1904).

[4] H. Wieland, "Über den Mechanismus der Oxidationsvorgänge, *Ber.*, **46**, 3339–40 (1913).

The application of this test was made by us under certain conditions which are to be considered standard for all experiments described, unless otherwise specified. To a test-tube containing 5 cc. of milk, or milk serum, were added a drop of salicylic aldehyde and 3 drops of methylene blue solution (concentration 1 g. per liter of water). The size of the drops was kept fairly constant by the use of a buret. The mixture was thoroughly shaken and the test-tube placed at once in a water bath kept at 65°, and the time of reduction of the dye was noted. From the deep blue color, the change proceeds through green to a faint yellow. The comparative times of reduction were used as standards for tests and conclusions.

The first plan of procedure included 4 steps: (1) the removal of the fat globules from the milk with the object of thinning the liquid as much as possible, thus making it suitable for convenient filtration; (2) the sterilizing of the milk solution by filtration through a germ-proof filter; (3) the sterilization of the fat, previously removed, by heat; (4) the subsequent mixing of the two sterilized fractions. This plan for sterilization should prove satisfactory, provided the enzyme content stays in the milk solution, instead of separating out with the fat.

The use of the word "milk" in what follows refers to whole milk. "Milk solution" means milk from which fat has been removed, and "milk serum" the filtrate in the filtration experiments.

Centrifugation was tried as a means of removing the fat. Milk was rotated in an electric centrifuge for 5 minutes, and the milk solution was then removed from the thick and partially hardened mass of fat above it by means of a pipet forced through the cream layer into the milk. This method of removing the milk solution from the centrifuge tube was not good, as particles of cream adhered to the pipet and became mixed with the solution. Later we used the method of Van Slyke[1] for the complete separation of fat from the milk solution, and obtained better results. To a liter of milk, 50 cc. of chloroform was added. Chloroform was found not to affect the activity of the Schardinger enzyme.[2] After being thoroughly mixed with the chloroform, the milk was centrifugated as described above. The chloroform layer containing the dissolved fat formed a hard mass at the bottom of the tube, from which the milk solutions was decanted.

Whole milk gave a reduction time of $7^1/_4 \pm {}^1/_4$ minutes; centrifugated milk $5^1/_4 \pm {}^1/_4$.

[1] L. L. Van Slyke, and A. W. Bosworth, "Condition of Casein and Salts in Milk," *J. Biol. Chem.*, **20**, 135 (1915).

[2] Chloroform had no effect upon the enzyme content, when used in the proportions indicated in preparing for centrifugation, *i. e.*, 50 cc. per liter. Milk treated with twice and with 4 times that amount, and allowed to stand for 4 hours with the chloroform, gave the enzyme test in the same length of time as fresh, untreated milk gave it.

The fact that the enzymes act more readily when the fat is not present cannot be definitely accounted for. Possibly the fat, when present, absorbs the salicylic aldehyde, and by so doing renders it less active as a reducing agent for the dye.

The separated cream, diluted with water, gave also an enzyme test, so it is certain that not all the enzymes of the milk remain in the milk solution after centrifugation. This would mean a loss of a small partof the enzyme content of the milk in the heat sterilization of the cream.

The first filter selected for trial, as absolutely germ-proof, was the Pasteur-Chamberland filter of porous clay and asbestos. The milk solution was filtered through it under pressure obtained by an apparatus illustrated in the accompanying diagram.

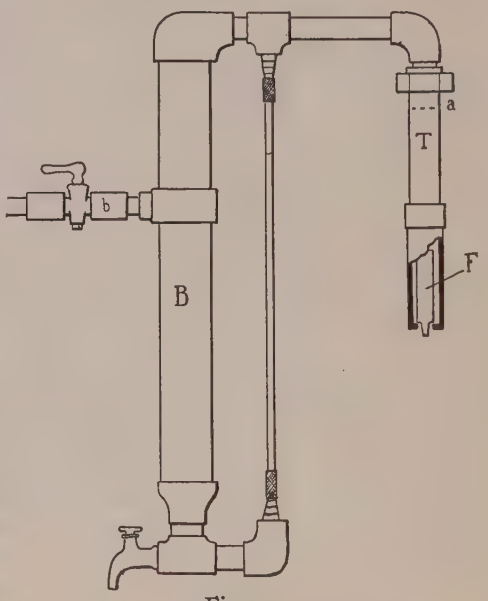

Fig. 1.

The iron tube T, above the filter F, was filled with milk solution to a level indicated by the dotted line *a*. The air in the remainder of the apparatus was compressed by allowing water to flow from the water main through the tube *b*, into the larger tube B. The pressure obtained was from 2–3 atmospheres, but even with such a pressure the filtration was slow. The filter became quickly clogged, and was found to be coated with a slimy deposit of casein.

The milk serum obtained by filtration was of a greenish color and slightly opalescent. An analysis of milk filtered under the same conditions has been published by Van Slyke.[1] He found that the content of the serum is not constant until enough of the milk solution has passed through the filter to saturate it thoroughly. He therefore states that the first 75 cc. of the filtrate must be discarded for this reason, but that the portion following contains constant quantities of its various constituents. The same principle was applied to the filtration of the milk for tests for enzymes. Even after filtering 200 to 500 cc. of the milk solution, so that the filter should be very thoroughly saturated, the serum gave not the faintest trace of an enzyme test. When the thick deposit on the surface of the filter was scraped off and suspended in water, this mixture

[1] *Loc. cit.*

was found to give the enzyme test, showing that the enzyme had remained with the casein.

The question then arose as to the possible disposition of the enzyme bodies in the milk solution. Are they attached to the casein molecule, and thereby prevented from passing through the filter, or are they free in solution? If the former condition exists, and the enzymes are tied to casein molecules, it would be impossible for them to pass through the filter, since all the casein remains on it, as shown by tests for casein on the filtrate by precipitation with acid. The filtrate showed no cloudiness upon the addition of acid in small quantity, therefore, no more than traces of casein could be present in it. The application of the biuret test showed the presence of protein, but this was given by the lactalbumin and unknown proteins of the milk which had passed through the filter. If the enzymes are free in solution, and the solution containing them could be separated from the casein by precipitation with acid, the casein-free solution with its enzyme content might be forced through the filter. The acid chosen for this precipitation must be one which does not destroy the enzymes.

If we add a very small amount of any acid to milk the Schardinger reaction takes place much more slowly, even if the amount is not sufficient for the precipitation of the casein.[1] If enough is added so that all the casein is precipitated, then the Schardinger reaction does not take place at all. There are, then, in relation to the Schardinger emzyme, three possibilities:

(1) The enzyme is still in *solution*. If we filter through ordinary filter paper, we can observe the Schardinger reaction as soon as we have neutralized the excess of acid which hindered the reaction.

(2) With stronger acid we can *precipitate* the enzyme so that it is mixed with the casein. We can now still obtain a positive Schardinger test, if we neutralize the acid carefully without filtering. If we filter, even through filter paper, the serum is quite inactive.

(3) By the addition of still stronger acid we can alter the enzyme so much that it is not possible to get any Schardinger test, neither in the serum nor in the casein. We consider then the enzyme as being *destroyed.*

In these experiments care must be taken not to add too much alkali, because in alkaline solution methylene blue is reduced by milk sugar, and this reduction could be mistaken for the reaction of Schardinger. Therefore we made sure that this point was not reached. If we neutralized without filtration from the casein, we used just the amount of sodium carbonate, which corresponded to the amount of acid used. If we had filtered from casein we had of course to use less sodium carbonate, because

[1] On the other hand a small amount of alkali increases the rapidity of the reaction.

casein is an acid itself. We added then sodium carbonate till the calcium salts of the milk began to be precipitated, that is, when the solution was about neutral towards litmus paper.

To avoid the possibility of a mistake, we made exactly the same operations with boiled milk and we never observed any decoloration of the methylene blue.

The amount of acid used for obtaining these 3 steps is not always absolutely constant. The first step was generally obtained by the addition of 0.4 cc. of saturated citric acid to 50 cc. of milk. (For the precipitation of the casein we need 0.3 to 0.4 cc. of citric acid. At the same time all the fat is precipitated with the casein, the fat emulsion being stable only in the colloidal casein solution.) The second step was observed after the addition of two or three times the stated amount of citric acid or after the addition of 0.4 cc. of 6 *N* sulfuric acid (29% H_2SO_4). The third step—destruction of the enzyme—was generally obtained by the addition of 2 cc. of citric acid or 0.8 cc. of sulfuric acid. Hydrochloric acid (6 *N*) seems to have considerably less action upon the enzyme than sulfuric acid.

In connection with the second step we might mention that it seems as if the enzyme could be separated by the acid into two compounds, the precipitate being inactive for itself if thoroughly washed with water, but becoming active again after mixing with the inactive serum. We have, however, not investigated this observation further.

After these preliminary experiments we precipitated the casein of one liter of milk according to step (1). Eight cc. of saturated citric acid were added. After filtration through paper and neutralization with sodium carbonate we obtained 100 cc. of a very active milk serum, the time for the Schardinger reaction being 1 min. 40 sec. to 1 min. 50 sec.

This milk serum passed through our Pasteur-Chamberland filter with ease, and the filter did not become clogged as before. But, unfortunately, the filtrate gave no trace of enzyme test, and again it was indicated, even more clearly than before, that the enzyme body is too large to pass through the pores of the filter, or that it is of a nature which does not permit its filtration under the conditions used.

The next filter tried was an asbestos felt, prepared from shredded asbestos, and arranged upon a Büchner funnel. The layer of asbestos was about a quarter of an inch thick. The filtrate from this gave the enzyme test, but the time of reduction of the dye was not constant until 100 cc. of solution had been filtered. When well saturated, the asbestos gave a filtrate in which the enzyme content was constant. Thus the final aim of the paper was partially attained, since a filtered solution of the enzymes was obtained. Such a filter, however, does not hold back bacteria, so a sterilized milk could not be obtained from it.

The last possibility was that of a Berkfeld filter. Its pores are larger than those of the Pasteur filters, and the Berkfelds are almost germ-proof. The milk solution, freed from fat and casein by precipitation with acid, was filtered with ease through a Berkfeld under pressure. But the filtrate gave no enzyme test, and after the filtration of about 1500 cc., there was still no indication that the enzyme had passed through.

It was suggested that in alkaline solution the enzyme might pass through the filter, the alkali changing the condition of the enzyme body in a way to make it possible to filter it. Accordingly, some active whey was made alkaline with sodium carbonate, and filtered. The filtrate was neutralized carefully with citric acid, and the enzyme test applied to the neutral solution, but with negative results.

Summary and Conclusions.—The chief purpose for which the work was carried on was not accomplished. No method for filtering the enzymes was found from which a bacteria-free filtrate was obtained. The main conclusion must, therefore, be negative. There are a few observations to be noted, and conclusions may be drawn tentatively from them, though the limited number of facts concerning enzymes known certainly, upon which to base conclusions, makes them rather of the nature of suggestions.

The Schardinger enzyme is found to be water-soluble. That is, it remains in the liquid portion of the milk after the casein and the fat are removed by a small amount of acid. If we precipitate the casein by stronger acids, the enzyme is precipitated with the casein and by still more acid it is destroyed.

It is possible that the enzyme is united in some way with some other water-soluble component of the milk, and that it remains stable and active only as long as this unknown body remains stable. Such a body might be an albumin. Van Slyke's table of analysis of milk serum shows that only 37% of the albumin passes through the filter. This, he says, may be due to the absorbing power of the casein molecules, which hold back the larger portion of it. If the enzymes are united to some albumin not passing through the filter, this union may account for their absence in the serum.

It is more probable, however, that they are separate bodies, having properties similar to those of the albumin, and of a closely allied nature. They remain active until the coagulation temperature of the lactalbumin is reached, 72–80°, and this is perhaps their own coagulation temperature.

The writer gratefully acknowledges the suggestions and aid of Professor Piccard in the completion of this work.

The work described in the first of this dissertation was carried out by Miss Rising as a war service in the Autumn of 1917 and Winter of 1918. The work reported in the second part was likewise intended to be a war contribution.

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